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(57) Abstract

An improved sandwich hybridisation diagnostic method in which there is used as the immobilised polynucleotide, a single-stranded polynucleotide sequence covalently coupled to particles or beads of a synthetic resin, preferably a cross-linked macroporous cellulosic resin. Also disclosed is a particular application of the method to the detection of the abnormal human (sickle cell) β-globin gene and diagnostic kits for use therein.

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IMPROVED SANDWICH HYBRIDISATION TECHNIQUE FOR THE DETECTION OF NUCLEOTIDE SEQUENCES

FIELD OF INVENTION

This invention relates to the detection of nucleotide sequences, including both DNA and RNA sequences.

BACKGROUND AND PRIOR ART

The ability to detect the presence (or absence) of a given RNA or DNA sequence in a sample, e.g. a clinically obtained sample or specimen, is potentially of great benefit to mankind. Existing methods of detection are, however, extremely time consuming and labour intensive and are therefore for use on anything other than a very limited scale. In many cases also sensitivity is undesirably low. In one commonly used procedure, for example, specific DNA sequences are detected in complex mixtures by digesting the sample with an appropriate restriction endonuclease, followed by size fractionation by gel electrophoresis, transfer of the sized fractions from the gel to a nitrocellulose or other suitable membrane, hybridisation to a radioactively labelled probe, usually a ^{32}P-labelled probe, and finally autoradiography. Such a procedure is inherently cumbersome and unsuited for automation. Also, very often the quantity of DNA or other nucleotide sequence to be detected and which is transferred onto the membrane is so small that in order to obtain a detectable signal after hybridisation a highly radioactive probe has to be used, which is disadvantageous both from the handling point of view and from the point of view that the radioactive label of choice, i.e. ^{32}P, has a relatively short half-life making it unsuitable for use as a stock reagent.

An alternative to the above is the sandwich hybridisation technique disclosed by Dunn A.R. and Hassell J.A. in (1977) Cell 12 23-26 in which the sample containing the nucleotide sequence to be detected is contacted with a complementary fragment immobilised on a membrane, e.g. a nitrocellulose filter, and to which the sequence to be detected hybridises in a first hybridisation step leaving an unhybridised tail available for a second hybridisation step with a labelled probe.

In EP-0079139 a proposal is made for the utilisation of the above described sandwich hybridisation technique in the identification of micro-

organisms in a sample. In accordance with that proposal a sample containing a single-stranded nucleic acid sequence from the micro-organism to be detected is contacted with two single-stranded fragments obtained from the genome of the micro-organism in question either directly or by recombinant DNA technology and complementary to different positions of the sequence to be detected, but not to each other, the one being immobilised on a solid carrier, preferably a nitrocellulose filter, and the other being labelled. Although labelling with labels other than radioactive labels is implied, only radioactive labelling is disclosed, and specifically labelling with ^{125}I. In the presence of the single-stranded nucleic acid sequence to be detected, which hybridises with both the immobilised fragment and the labelled fragment, the labelled fragment becomes bound to the carrier and can be detected thereon by autoradiography, thus giving rise to positive identification of the micro-organism from which that single-stranded sequence originated.

Whilst the sandwich hybridisation technique provides advantages over the first described procedure inherent disadvantages remain arising particularly from the finite and quite limited quantity of nucleic acid which can be immobilised onto the nitrocellulose filter or other membrane. This in particular places limits on both the speed and sensitivity of the detection method. Also it appears that the efficiency of the hybridisation particularly in the second stage is very low, thus reducing the sensitivity of the method still further.

In EP-0070687 a hybridisation diagnostic method is disclosed which uses light-labelled single-stranded polynucleotide reagents for hybridising with immobilised sample single-stranded polynucleotides. A variety of solid supports are suggested for the immobilised sample single-stranded polynucleotide including activated glass beads, polyacrylamide, agarose or sephadex beads, and cellulose. Various known methods are also suggested for coupling the sample polynucleotide to the support, largely by reference to published literature, e.g. Methods in Enzymology Vol. XXXIV, Part B, 463-475, 1974 and Vol. XLIV, 859-886, 1976, but no specific examples of an operative method are given. It is also suggested that the sample single-stranded polynucleotide can be immobilised by contacting the sample under hybridisation conditions with an immobilised first single-stranded polynucleotide reagent complementary to a different and mutually exclusive portion of the sample polynucleotide to that which is required for the

hybridisation of the light-labelled polynucleotide reagent, i.e. a so-called sandwich hybridisation procedure. Again no specific examples are given of the procedure, or of the method by which the first polynucleotide reagent is attached to the support in the first place.

SUMMARY OF THE INVENTION

In accordance with one aspect of the present invention we have discovered that both the speed and efficiency of the sandwich hybridisation reaction, particularly the second stage, can be substantially increased by using solid particles or beads of a macroporous resin as the carrier for the immobilised nucleic acid fragment thus providing a method for the detection of nucleic acid fragments which is capable of providing a much improved sensitivity. In particular the use of particles or beads of a cross-linked macroporous resin such as sephacryl appears to result in substantially improved covalent bonding of the polynucleotide reagent to the support.

In a second aspect of this invention, a particularly sensitive method has been developed for the detection of the abnormal human (sickle cell) β-globin gene by the sandwich hybridisation of a Dde I digest of the abnormal gene with an immobilised polynucleotide reagent covalently bonded to solid resin particles or beads, and a second labelled polynucleotide reagent, such reagents comprising polynucleotide sequences obtained by restriction endonuclease treatment of the normal gene and complementary to different portions of a restriction fragment contained within the digest and containing the single base change in the sixth codon of the β-globin gene that is characteristic of the abnormal (sickle cell) β-globin gene.

In accordance with the present invention therefore, there is provided a method for the detection of a given nucleic acid sequence in a sample, which comprises contacting the sample under hybridisation conditions with a first reagent comprising solid particles or beads having immobilised thereon a single-stranded nucleic acid fragment comprising a sequence complementary to a portion of the sequence to be detected and with a second reagent comprising a labelled single-stranded nucleic acid fragment comprising a sequence complementary to a different portion of the sequence to be detected, but non-complementary to the immobilised fragment, thereby forming a hybridisation duplex comprising the sequence to be detected hybridised with both reagents, separating the hybridisation duplex from any

remaining unbound label, and detecting the presence, if any, of label bound to the hybridisation duplex.

DETAILED DESCRIPTION

The first reagent used in the method of this invention preferably comprises a single-stranded nucleic acid fragment immobilised onto the solid particles or beads by covalent bonding between reactive, e.g. amino, groups in the nucleic acid fragment, and reactive groups in or on the solid particle. To this end the preferred solid particles used in the method of this invention are polymer beads formed from a natural or synthetic polymer having reactive, e.g. amino or hydroxy, groups attached to the polymer chain. Most preferred are cellulosic polymer beads such as those sold under the trade names Sephadex and Cellex and more particularly macroporous cellulosic materials such as Sepharose and Sephacryl.

Various methods exist and are known for the covalent bonding of nucleic acid fragments to such polymer beads, including coupling with carbodiimides via terminal phosphate groups and coupling via cyanogen bromide. Particular mention may be made, however, of coupling via diazotisation of aromatic amino groups attached to the polymer matrix, which technique is disclosed in detail in (1982) Nucleic Acid Res. 10, 22, 1799-1810 and 7163-7196.

Preferably the polymer beads will have a particle size in the range 5 to 50 microns, more preferably 10 to 20 microns.

Likewise various methods exist and are known for the labelling of single-stranded nucleic acid fragments to provide the second reagent used in the method of this invention. Labelling with radioactive isotopes such as ^{32}P and ^{125}P has already been mentioned and can be used in the present invention. However, it is envisaged that, because of the greater sensitivity that can be achieved by the present invention, less highly radioactive isotopes can be used as the label and in particular tritium. Also it is envisaged that non-radioactive labels e.g. enzymatic, fluorescent and chemiluminescent labels will be suitable, and in many cases may be preferred.

Once the reagents have been prepared, and it is envisaged that these will be available in kit form, the method of the invention is extremely simple to carry out and is readily adaptable to automatic or semi-automatic equipment. Essentially it is necessary merely to mix the sample with the two

reagents, either simultaneously or successively, under conditions effective to permit formation of the hybridised duplex comprising the two reagents hybridised to the restriction fragment which is to be detected. The hybridisation is rapid, and since in contrast to the sandwich hybridisation technique of EP-0079139, both the first (immobilised) reagent and the second (labelled) reagent can be used to infinite excess, merely by increasing the quantity used, the amount of bound label can be maximised thereby to provide a maximum signal for subsequent detection. Thus the method of the present invention can be of great sensitivity, this being determined not by the concentration of the reagents, which effectively can be infinite, but by the concentration of the sequence to be detected in the sample. This is in marked contrast to the method of EP-0079139 where the concentration of the immobilised reagent is finite, and relatively low, so that the quantity of label bound to the duplex is equally low and is determined not by the concentration in the sample of the sequence to be detected, which is usually far in excess of the quantity of the immobilised reagent, but by the quantity of the immobilised agent. In the method of the present invention a far higher proportion of the sequence to be detected present in the sample is hybridised and consequently labelled. Not only that, but as already indicated, there is a substantial and surprising increase in the efficiency of the hybridisation reaction using beads as the carrier as opposed to a filter or membrane, e.g. of cellulose nitrate.

The sample used in the method of this invention can be a purified and/or fractionated sample containing the polynucleotide fragment or fragments to be identified in single-stranded form, but it is a major advantage of this invention that the sequence to be detected does not have to be separated from the sample prior to labelling as in the Southern blotting technique. It is therefore possible in accordance with the present invention to detect a given polynucleotide sequence in a crude mixture of polynucleotide fragments obtained by the digestion of the original sample, e.g. a clinical sample containing DNA or RNA, with an appropriate restriction endonuclease and subsequent denaturation to reduce the restriction fragments to single-stranded form.

Following hybridisation the polymer beads or other particles carrying the labelled hybridisation duplex are separated, e.g. by centrifuging and washing, from unbound excess label and the presence of bound label detected

in the appropriate manner, e.g. by a scintillation counter in the case of a radioactive label, or by enzymatic activity in the case of an enzyme label, or light detecting means in the case of a fluorescent or chemiluminescent label.

The method of the present invention has a wide range of applications arising from the ability rapidly to identify particular nucleic acid (DNA or RNA) sequences in a sample. Thus it can be used to identify pathogenic micro-organisms in a sample, genetic abnormalities, etc. as well as simply a gene mapping exercise. A particular application given by way of example is in the identification of sickle cell disease.

Sickle cell disease is caused by a single base mutation (A → T) in the sixth codon of the human β-globin gene. This change from GAG to GTG in the sixth codon, besides altering the properties of the resultant haemoglobin coded for by the gene with valine being incorporated into the protein rather than glutamine, also results in the loss of the Dde I and Mst II recognition sites at the 5' end of the β-globin gene. This is diagramatically illustrated in the accompanying drawing which is a restriction map of Dde I and Hinf I sites at the 5' end of the β-globin gene. Dde I digestion of abnormal (sickle cell) β-globin gene will therefore produce a single 381 bp fragment comprising both Fragments B and C in a single continuous length, as opposed to two separate fragments, one of 201 bp (Fragment B) and 180 bp (Fragment C), that will be produced by Dde I digestion of the normal β-globin gene. Using the techniques already described to immobilise one of Fragment B or Fragment C onto polymer beads and to label the other, two reagents are provided capable of rapidly detecting the presence of abnormal sickle cell gene in a sample. Thus digestion of normal β-globin gene with Dde I will produce two fragments neither of which is capable of forming a "sandwich" with the two reagents. On the other hand, digestion of abnormal (sickle cell) β-globin gene with Dde I will produce a single fragment which is capable of forming a "sandwich" with the two reagents. When the Dde I digest is contacted with the two reagents under hybridisation conditions (i.e. after denaturation of the digest) the abnormal (sickle cell) β-globin gene will result in binding of the label, and hence a positive signal, whereas the normal β-globin gene will not.

In a practical experiment, the above procedure has been mimicked by digesting plasmid βF5 with Hinf I. Plasmid βF5 contains the 1.9 kb Bam HI restriction fragment of the human β-globin gene cloned into the single Bam

HI site of the vector pAT 153. Digestion of the plasmid βF5 with Hinf I produced a 341 kb fragment (Fragment A) which was recovered from the product of digestion by electrophoresis and electroelution.

After denaturation the 341 kb fragment was immobilised onto macroporous cellulosic polymer beads (Sephacryl S500) using the diazotisation method described by Seed B. in (1982) Nucleic Acids Res. 10, 1799-1810.

In a second operation plasmid βF5 was digested with Mst II to produce a 201 bp fragment (Fragment B, see Figure 1) which was likewise recovered from the digest. Fragment B was then labelled with ^{32}P by nick translation using the technique of Rigby et al. (1977) J. Mol. Biol. 113, 237-251, to a specific activity of 1.5×10^7 cpm μg^{-1}.

In two further operations plasmid βF5 was digested with Dde I and Bam HI. Digestion with Dde I produces short fragments not capable of "sandwich" hybridisation with Fragments A and B, whereas digestion with Bam HI produces one long fragment which is capable of "sandwich" hybridisation. In each case digestion was followed by denaturation to provide single-stranded DNA fragments.

Prior to sandwich hybridisation by the technique of the present invention the immobilised Fragment A was prehybridised overnight at 65°C in 1 ml. 40 mM PIPES pH 6.5, 1 mM EDTA 0.6 M NaCl, 0.1% SDS and 250 $\mu g\ ml^{-1}$ sonicated denatured salmon sperm DNA. Following prehybridisation, sandwich hybridisation was carried out by mixing either the denatured Dde I or Bam HI βF5 digests with immobilised 341 bp Fragment A and labelled 201 bp Fragment B in 50 µl of the same buffer and allowing the mixture to stand overnight. The beads were then separated, washed three times for 10 minutes each at 65° with 1 ml of the same buffer but without the salmon sperm DNA and then counted. The results are shown in Table 1. These clearly show that with amounts of plasmid βF5 of 100 attomoles or over, a clear distinction can be drawn between the Bam HI digest and the Dde I digest. In the figures given in Table 1 the low background count obtained when either the "sandwich filling" was omitted or replaced by sonicated calf thymus DNA has not been subtracted.

Table 1

Amount of restricted βF5 plasmid (attomoles)	cpm on resin when enzyme is Bam HI	cpm on resin when enzyme is Dde I
10,000	552	63
5,000	748	70
2,000	482	65
1,000	266	71
500	196	44
100	107	62
80	90	61
60	82	60
40	62	57
20	75	60

In a further practical experiment, the above sandwich hybridisation using the immobilised 341 bp Fragment A and the labelled 201 bp Fragment B has been repeated using a Dde I digest of 60 attomoles of human DNA both from a normal patient and from a homozygote sickle patient. The results were unequivocal. After background substraction, a typical experiment gave 441 counts (10 minutes) for sickle DNA and 153 counts for normal DNA illustrating and confirming the effectiveness of the method of the invention in identifying the presence of sickle DNA.

By mixing the Bam HI and Dde I digests of βF5 it is possible also to mimic the case of the sickle heterozygote. In such an experiment, counts (10 minutes) were obtained using 100 attomoles plasmid DNA as follows: normal 128; heterozygote 296; homozygote 745; thus indicating that the method can be quantitated to identify heterozygotes as well as homozygotes.

Im summary, the method of the present invention has the following advantages over present technology:

1. It is quicker. Electrophoresis, blotting and autoradiography are eliminated. The sandwich hybridisation can be carried out in as little as 8

hours; the separation of beads by gravity, centrifuge or magnetically takes seconds; and quantitation in a scintillation counter takes a few minutes. In practice results have been obtained within 48 hours of taking blood and the indications are that substantial reductions will be possible in the future.

2. It is extremely easy to automate and hence to process a large number of samples.

3. It is readily adaptable to other methods of DNA labelling.

4. It works as well with short restriction fragments as with longer ones and is readily adaptable for the use of oligonucleotides. This greatly enlarges the number of potential applications.

5. Whereas membranes have a (low) finite capacity for binding DNA, larger quantities of DNA can be readily handled in the present procedure by simply increasing the quantity of immobilised and labelled DNA's. This offers the possibility of either increasing the sensitivity of the assay or of using a labelled DNA of lower specific activity or of reducing the hybridisation time still further.

These advantages will now permit the rapid adoption of recombinant DNA technology to routine laboratories for the identification and characterisation of bacteria, viruses, and genetic disorders in humans, animals and plants.

CLAIMS

1. In a method for the detection of a given nucleotide sequence in a target polynucleotide, which comprises contacting the target polynucleotide under hybridisation conditions with (a) an immobilised polynucleotide comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a first section of said sequence to be detected, and (b) a labelled polynucleotide probe comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a second and different section of the sequence to be detected, labelled with a marker, thereby to form an immobilised hybridisation duplex comprising the immobilised polynucleotide, the target polynucleotide and the labelled polynucleotide, separating the immobilised duplex and determining the presence of the marker thereon, the improvement which comprises using as said immobilised polynucleotide, a polynucleotide containing said single-stranded complementary nucleotide sequence covalently bonded to solid particles or beads of a cross-linked macroporous resin.

2. In a method for the detection of a given nucleotide sequence in a target polynucleotide, which comprises contacting the target polynucleotide under hybridisation conditions with (a) an immobilised polynucleotide comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a first section of said sequence to be detected, and (b) a labelled polynucleotide probe comprising a single-stranded nucleotide sequence complementary to, and hybridisable under said conditions, with a second and different section of the sequence to be detected, labelled with a marker, thereby to form an immobilised hybridisation duplex comprising the immobilised polynucleotide, the target polynucleotide and the labelled polynucleotide, separating the immobilised duplex and determining the presence of the marker thereon, the improvement which comprises using as said immobilised polynucleotide, a polynucleotide containing said single-stranded complementary nucleotide sequence covalently bonded to solid particles or beads of a cross-linked macroporous cellulosic material, said particles or beads having a particle or bead size in the range 5 to 50 μ.

3. A method according to claim 2, wherein the immobilised polynucleotide

is covalently bonded to the resin particles or beads by a diazotisation reaction between reactive groups on the resin and free amino groups on the polynucleotide.

4. A method according to claim 2, wherein the immobilised polynucleotide is covalently bonded to the resin particles or beads by carbodiimide coupling between the phosphate groups at the 5' end of the polynucleotide and reactive groups on the resin.

5. A method according to claim 2, wherein the polynucleotide probe comprises a radioactive marker or label.

6. A method according to claim 2, wherein the polynucleotide probe comprises an enzyme marker or label.

7. A method according to claim 2, wherein the immobilised polynucleotide and the polynucleotide probe are restriction endonuclease polynucleotide fragments.

8. A method for the detection of the abnormal human (sickle cell) β-globin gene, which comprises digesting the gene with a Dde I restriction endonuclease to produce a restriction digest containing as a component a 381 bp restriction fragment containing the abnormal sixth codon which is a characteristic of the abnormal gene, contacting the digest containing that fragment under hybridisation conditions with (a) an immobilised single-stranded polynucleotide covalently bonded to solid resin particles or beads, said single-stranded polynucleotide being hybridisable with a first section of said fragment, and (b) with a labelled single-stranded polynucleotide probe hybridisable with a second section of said fragment, wherein one of said single-stranded polynucleotide probe and said labelled single-stranded polynucleotide is or comprises a 180 bp polynucleotide sequence obtained or obtainable by Dde I digestion of normal human β-globin gene and complementary to a first section of said 381 bp fragment, and the other is or comprises a 201 bp fragment obtained or obtainable by Dde I digestion of normal β-globin gene and complementary to a second, different, section of said 381 bp fragment, separating the resulting immobilised hybridisation duplex comprising the

immobilised single-stranded polynucleotide, the said restriction fragment, and said labelled polynucleotide probe, and determining the presence of the marker on the immobilised hybridisation duplex.

9. A method according to claim 8, wherein the said 180 bp polynucleotide sequence is part of a 341 bp fragment obtained or obtainable by Hinf I digestion of normal human β-globin gene.

10. A method according to claim 8, wherein the polynucleotide consisting of or containing said 180 bp sequence, as the case may be, and the polynucleotide comprising said 201 bp fragment are obtained by the endonuclease restriction of plasmid βF5.

11. A method according to claim 10, wherein the immobilised single-stranded polynucleotide is covalently coupled to beads or particles of a cross-linked macroporous cellulosic resin.

12. A diagnostic kit for the detection of a given nucleotide sequence in a target polynucleotide, comprising as a first component an immobilised polynucleotide reagent comprising solid particles or beads of a cross-linked macroporous resin having a single-stranded polynucleotide sequence complementary to a first section of the nucleotide sequence to be detected covalently bonded to said particles or beads, and as a second component, a polynucleotide probe comprising a single-stranded nucleotide sequence complementary to a second, different section of the nucleotide sequence to be detected, and labelled with a marker.

13. A diagnostic kit for the detection of the abnormal human (sickle cell) β-globin gene, comprising as a first component an immobilised polynucleotide reagent comprising solid resin particles or beads having covalently bonded thereto a first single-stranded polynucleotide sequence, and as a second component a polynucleotide probe comprising a second single-stranded polynucleotide sequence labelled with a marker, wherein one of said sequences is or comprises said 180 bp polynucleotide sequence obtained or obtainable by Dde I digestion of normal human β-globin gene, and the other is or comprises said 201 bp fragment obtained or obtainable by Dde I digestion of normal β-globin gene.

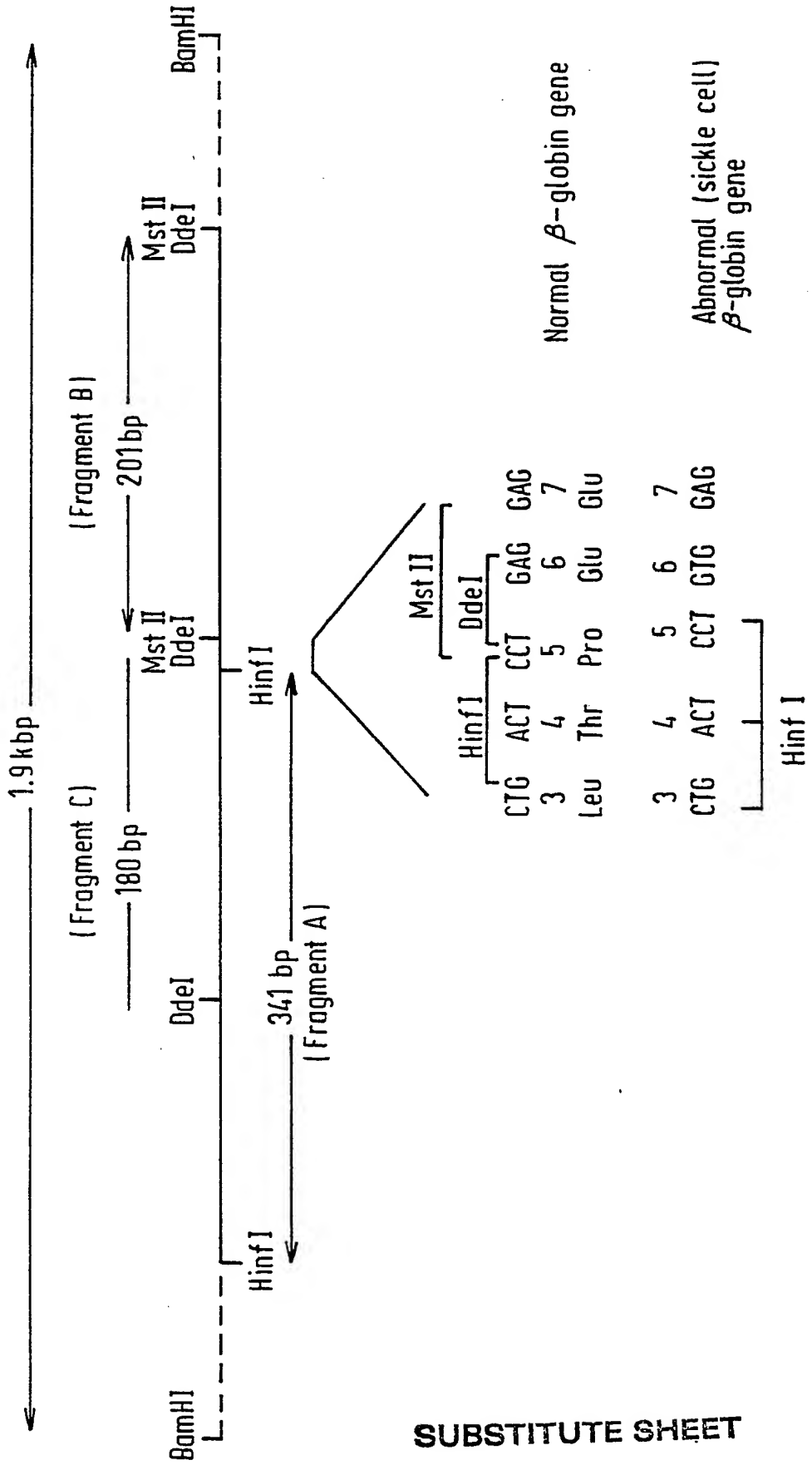
14. A kit according to claim 13, wherein the said 180 bp polynucleotide sequence is part of a 341 bp fragment obtained or obtainable by Hinf I digestion of normal human β-globin gene.

15. A kit according to claim 13, wherein the polynucleotide consisting of or containing said 180 bp sequence, as the case may be, and the polynucleotide comprising said 201 bp fragment are obtained by the endonuclease restriction of plasmid βF5.

16. A kit according to claim 13, wherein said first single-stranded polynucleotide sequence is covalently coupled to particles or beads of a cross-linked macroporous resin.

17. A kit according to claim 12, wherein the polynucleotide probe is labelled with an enzyme, and the kit also contains, as a third component, means for detecting the enzyme-labelled probe, said means comprising a substrate for said enzyme.

18. A kit according to claim 13, wherein the polynucleotide probe is labelled with an enzyme, and the kit also contains, as a third component, means for detecting the enzyme-labelled probe, said means comprising a substrate for said enzyme.


BamHI
1.9 kbp
BamHI
(Fragment C)
180 bp
(Fragment B)
201 bp
Mst II
DdeI
Mst II
DdeI
DdeI
Hinf I
341 bp
(Fragment A)
Hinf I
Mst II
DdeI
Hinf I
CTG ACT CCT GAG GAG
3 4 5 6 7
Leu Thr Pro Glu Glu
Normal β-globin gene
3 4 5 6 7
CTG ACT CCT GTG GAG
Abnormal (sickle cell) β-globin gene

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00591

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: C 12 Q 1/68 // G 01 N 33/546 // G 01 N 33/548

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	C 12 Q G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [10]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0070687 (STANDARD OIL COMPANY) 26 January 1983, see page 4, lines 4-24; page 8, line 29 - page 9, line 32; claims 1,2 (cited in the application)	1,2,12
P,Y	EP, A, 0130523 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see pages 1,2; page 8, line 31 - page 9, line 2; example	1,2,5,8-16
P,Y	EP, A, 0130515 (MOLECULAR DIAGNOSTICS, INC.) 9 January 1985, see the whole document	1,2,5,8-16
A	US, A, 4395486 (L.B. WILSON et al.) 26 July 1983, see the whole document	8-10,13-15
A	Proceedings of the National Academy of Science USA, volume 80, January 1983 B.J. Conner et al.:"Detection of sickle cell β^S-globin allele by hybridization with synthetic oligonucleotides",	./.

* Special categories of cited documents: [10]

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
2nd April 1986	24 AVR. 1986
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	M. VAN MOL

International Application No. PCT/GB 85/00591

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	see pages 278-282	8-10,13-15
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A	EP, A, 0079139 (ORION CORPORATION LTD.) 18 May 1983, see the whole document (cited in the application)	1,2,5
	--	
A	WO, A, 84/04332 (NATIONAL RESEARCH DEVELOPMENT CORPORATION) 8 November 1984, see the whole document	1,2,5,6
	--	
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